Bystander Effect in Gene Therapy of Chondrosarcoma Using Suicide Gene

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**Introduction:** Chondrosarcoma is second to osteogenic sarcoma in frequency as a malignant tumor of bone. It is difficult to obtain wide surgical resection margins because of its location adjacent to neurovascular and visceral structures. Neither irradiation nor chemotherapy seems to be an effective treatment for chondrosarcoma; thus new protocols are needed. We reported gene therapy of chondrosarcoma using retrovirus vector encoding suicide gene at the 45th annual meeting of ORS. Our model system using gene therapy demonstrated that the strong bystander tumoricidal effect was shown in vitro and tumor size was reduced markedly in vivo. The mechanism of bystander effect is still unclear. Metabolic cooperation mediated by the gap junction is suggested as a major contributor to the phenomenon. However, chondrosarcoma cells contain an extracellular matrix, which may inhibit the transport of substrates from cells to cells. In order to clarify the mechanism of the bystander effect, we established a 3-dimensional cell culture system, simulating chondrosarcoma in vivo. In this study, the bystander effect was investigated in cell culture system and in vivo tumor.

**Materials and Methods:** Tumor cells and animals: Human chondrosarcoma cell line HCS-TG was used. Male ICR nu/nu nude mice were used to determine in vivo experiments.

**Production of recombinant retrovirus vectors and retrovirus Infection:** We produced recombinant retrovirus vectors (pLTRNL) bearing a herpes simplex virus thymidine kinase (HSV-tk) gene. The strategy is to infect chondrosarcoma cells by virtue of HSV-tk gene expression, and to sensitize them to antitherpetic drug, ganciclovir (GCV). Ganciclovir is a guanosine analog, which is metabolized to a cytotoxic product by HSV-tk. In the other vector (LZRNL) the HSV-tk gene was replaced by Escherichia coli beta-galactosidase gene (lacZ gene). To produce a transmissible virus, vector DNA was transfected with the calcium phosphate coprecipitation method into amphotropic producer cells (PA317). The human chondrosarcoma cells (HCS-TG) were plated in 10 cm dishes and infected 24h later by exposure for 48h to virus from the PA317/LTRNL vector-producer line or the PA317/LZRNL in the presence of 8µg/ml polybrene. These were cultured with previous medium containing the G418 (neomycin analogue). The G418-resistant clones of HCS-TG/LTRNL (HCS-TG-tk) and HCS-TG/LZRNL (HCS-TG-Z) were selected randomly from the surviving colonies and used in the following experiments.

**Collagen Gel Embedded Culture method:** 3-dimensional cell culture using collagen gel was applied in this study. Three layers were made in 3.5cm dishes. Base layer was made of Cellmatrix Type I A (derived from pig type I collagen), RPMI 1640 and HEPES in 3.5 cm dishes. Top layer was made of HCS-TG cells embedded with collagen gel and piled up on base layer. Overlay medium of RPMI 1640 was added.

**GCV sensitivity and bystander effect:** The cytotoxicity of the nucleoside analog ganciclovir (GCV) was determined by using a tetrazolium-based colorimetric assay (MTT assay). We examined in vitro bystander tumoricidal effect by coculturing with HCS-TG-Z and HCS-TG-tk. For cell survival analysis in coculture, cells were quantitated directly on culture plates after staining for X-gal activity. Tumor cell lines HCS-TG-tk and HCS-TG-Z were cocultured respectively at a moderate density in medium containing 10 µM GCV for 5 days. Further, tk- cells were cocultured with tk+ cells (lacZ+) at ratios of 1:1, 2:1, 1:2, 1:5, 1:10, 1:20, 1:50 for a total of 3×10⁵ cells per 35-mm well.

**In vivo experiments:** Chondrosarcoma was implanted in 20 nude mice. When the tumors grew to approximately 250 mm³ (6×6×7 mm³), the animals were divided into two groups. Ten tumors were injected with 1×10⁶ HCS-TG-tk cells subcutaneously. An additional 10 tumors were injected in a similar manner with HCS-TG-Z cells subcutaneously. One week after injection, nude mice were injected twice daily intraperitonelally for 14 days with GCV at 150 mg/kg of body weight. Tumor size was measured with calipers twice a week from day 0 (first ganciclovir treatment) to day 28. On day 14, some chondrosarcoma tumors injected with HCS-TG-tk and HCS-TG-Z were resected and stained with H.E. and X-gal.

**Results:** GCV sensitivity and bystander tumoricidal effect: The cytotoxic activity of GCV was dose-dependent in the HSV-tk gene-transduced clones of the human chondrosarcoma cell line. However, no effect was shown in cells without gene transduction. 97% of the gene transferred cells were killed at the concentration of 10 µM of GCV, and 90% of the cells at the concentration of 10 µM (Fig.1). The cytotoxic activity of GCV was observed in 3-dimensional cell culture. We observed the bystander effect at various cell ratios in cocultures of HCS-TG-tk and HCS-TG-Z. At a ratio of HCS-TG-tk: HCS-TG-Z cells of 1:10, 80% of cells were killed (Fig 2). This experiment showed strong bystander effect in 3-dimensional cell culture.

**In vivo inhibition of HCS-TG chondrosarcoma growth by local injection of HCS-TG-tk+ cells and treatment with GCV:** On day 28 the mean tumor size treated with HCS-TG-tk cells and GCV was one-fourth that treated with HCS-TG-tk and GCV (Fig 3). Significant bystander tumoricidal effects on chondrosarcoma growth rate were observed (P<0.01). On day 14 tumor necrosis was found in the group with HCS-TG-tk cells and GCV histologically. Necrotic area in the tumor was becoming wider with time after injection of HCS-TG-tk cells, with GCV treatment.

**Conclusion and discussion** Understanding the mechanism of the bystander effect is very important of cancer gene therapy. It has been emphasized that gap junctional communication plays an important role in the bystander effect. In this study, however, the bystander effect was observed even in 3-dimensional cell culture without gap junction. The gap junction was not always necessary to the bystander effect. So it has shown that another mechanism such as apoptosis, endocytosis of toxin cell debris, soluble toxins have been worked.